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ORIGINAL ARTICLE

Comparative evaluation of IGF-I gene transfer and IGF-I protein administration for enhancing skeletal muscle regeneration after injury

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Developing methodologies to enhance skeletal muscle regeneration and hasten the restoration of muscle function has important implications for minimizing disability after injury and for treating muscle diseases such as Duchenne muscular dystrophy. Although delivery of various growth factors, such as insulin-like growth factor-I (IGF-I), have proved successful in promoting skeletal muscle regeneration after injury, no study has compared the efficacy of different delivery methods directly. We compared the efficacy of systemic delivery of recombinant IGF-I protein via mini-osmotic pump (~1.5 mg/kg/day) with a single electrotransfer-assisted plasmid-based gene transfer, to hasten functional repair of mouse tibialis anterior muscles after myotoxic injury. The relative efficacy of each method was assessed at 7, 21 and 28 days post-injury. Our findings indicate that IGF-I hastened functional recovery, regardless of the route of IGF-I administration. However, gene transfer of IGF-I was superior to systemic protein administration because in the regenerating muscle, this delivery method increased IGF-I levels, activated intracellular signals (Akt phosphorylation), induced a greater magnitude of myofiber hypertrophy and hastened functional recovery at an earlier time point (14 days) after injury than did protein administration (21 days). Thus, the relative efficacy of different modes of delivery is an important consideration when assessing the therapeutic potential of various proteins for treating muscle injuries and skeletal muscle diseases.
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Introduction

Injury to skeletal muscles by external factors (e.g. crush, laceration, contusion, surgery) or internal factors (e.g. strains) can result in significant loss of function that can dramatically affect quality of life. Although skeletal muscle has the ability to regenerate after injury, functional repair can be slow, inefficient and incomplete. Thus, developing therapeutic approaches to enhance skeletal muscle regeneration and hasten restoration of muscle function is critical for improving the long-term physical outcome of patients suffering muscle injuries and for preventing or minimizing functional disability.[1] In addition, enhancing regeneration has therapeutic potential for various myopathies, including the muscular dystrophies and inflammatory muscle diseases.

The cellular and molecular mechanisms of muscle repair and regeneration have been described extensively.[2] In addition to the tightly controlled induction of myogenic regulatory factors and other muscle-specific genes, muscle injury and subsequent repair processes induce the release of various biologically active molecules that are critical for regeneration. These secreted factors include, but are not limited to, hepatocyte growth factor, fibroblast growth factors, leukemia inhibitory factor and insulin-like growth factors (IGFs).[3–7]

Insulin-like growth factor-I (IGF-I) is particularly relevant given that levels are elevated after various types of injury, during the formation of new fibers or the growth of existing fibers.[8] Increased levels of IGF-I *in vitro* alter the magnitude and timing of myogenic regulatory factors and other cell cycle-related proteins involved in muscle regeneration.[9] *In vivo*, IGF-I increases both satellite cell proliferation and the rate of protein synthesis of existing muscle fibers.[10,11] The signals induced by IGF-I ultimately lead to increased proliferation, differentiation and fusion of myoblasts and myotube/myofiber hypertrophy.[12–14]

Consequently, a basis exists for the administration of IGF-I to enhance muscle regeneration and promote functional recovery after injury. However, no study has directly compared different methods for increasing IGF-I levels during muscle regeneration. Studies on transgenic mice have shown that IGF-I overexpression prevents an age-related decrease in skeletal muscle mass and regenerative capacity and that IGF-I enhances skeletal muscle regeneration after myotoxic injury or nerve damage.[15–17] Viral-mediated IGF-I delivery prevents the age-related loss of skeletal muscle function and has been shown to promote skeletal muscle hypertrophy in an additive manner when combined with resistance exercise

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training.[18,19] Non-viral plasmid-based electroporation-assisted IGF-I gene transfer has been shown to enhance skeletal muscle regeneration after laceration injury.[20] Administration of recombinant IGF-I protein has been shown to promote restoration of muscle function after strain or laceration injuries.[21,22]

Administration of recombinant protein is a facile method, but is often hindered by short half-lives of the protein or the high expense of delivering an adequate amount of the protein. Non-viral gene transfer is of interest because it is inexpensive, can accommodate larger transgenes and is associated with a lower cytotoxicity compared to viral-mediated gene delivery. Although non-viral gene transfer can be limited by low transfection efficiency or local tissue damage, we have successfully resolved some of these issues.[23] Our aim in this study was to compare this methodology with recombinant protein administration in a model of muscle regeneration, and test the hypothesis that non-viral gene transfer of IGF-I was more effective for enhancing muscle repair and restoring muscle function after myotoxic injury.

Results

Histology and immunofluorescence

Our objective was to assess the effect of increased IGF-I levels on the time course of muscle regeneration, and so we transfected the muscle at the earliest time point after injury. Enhanced green fluorescence protein (EGFP) fluorescence was substantially higher in transverse sections of tibialis anterior (TA) muscles following plasmid injection and electrotransfer 4 days after compared to 2 or 3 days after the notexin-induced injury (Figure 1). Existing myofibers were still undergoing degradation 2 days after notexin injection and complete degeneration of the myofibers occurred 3 days after notexin injection (Figure 1). Importantly, the emergence of new myotubes (or immature myofibers) 4 days after notexin injection coincided with a dramatic increase in the transfection efficiency, as visualized by EGFP fluorescence (Figure 1).

We have previously demonstrated that electrotransfer enhances transfection efficiency in uninjured mouse TA muscle.[23] Given that transfection efficiency of naked plasmid DNA injection is higher in regenerating muscle compared to uninjured muscle, and rivals the transfection efficiency of adenoviral infection, we determined whether the electrotransfer was redundant in regenerating muscle.[24–27] The area of EGFP fluorescence was greater in transverse sections of regenerating TA muscles following electrotransfer-assisted plasmid gene transfer compared to naked plasmid DNA injection (Figure 1). Therefore, our results showing enhanced transfection efficiency in regenerating muscle indicate that electrotransfer was performed at the appropriate time after injury. In addition, we have demonstrated that electroporation-assisted gene transfer resulted in plasmid penetration along the entire muscle length (Supplementary Figure 1).

Representative photomicrographs and graphical representations of TA muscle fiber cross-sectional area (CSA) are presented in Figure 2. The median CSA of myofibers was not different for all conditions 7 days after notexin-induced injury. Fourteen days post-injury, the median CSA of myofibers was significantly greater with electrotransfer of the plasmid encoding IGF-I (1750–1821 μm^2; 95% confidence interval (CI) of the median; $n = 2423$ fibers) compared to protein delivery (1515–1595 μm^2; $n = 1914$ fibers) or electrotransfer with pcDNA3.1 (1295–1351 μm^2; $n = 2421$ fibers). Similarly, 21 days post-injury, the median CSA of myofibers was significantly greater with electrotransfer of the plasmid encoding IGF-I (2254–2353 μm^2; $n = 2266$ fibers) compared to protein delivery (1982–2190 μm^2; $n = 1930$ fibers) or electrotransfer with pcDNA3.1 (1645–1721 μm^2;

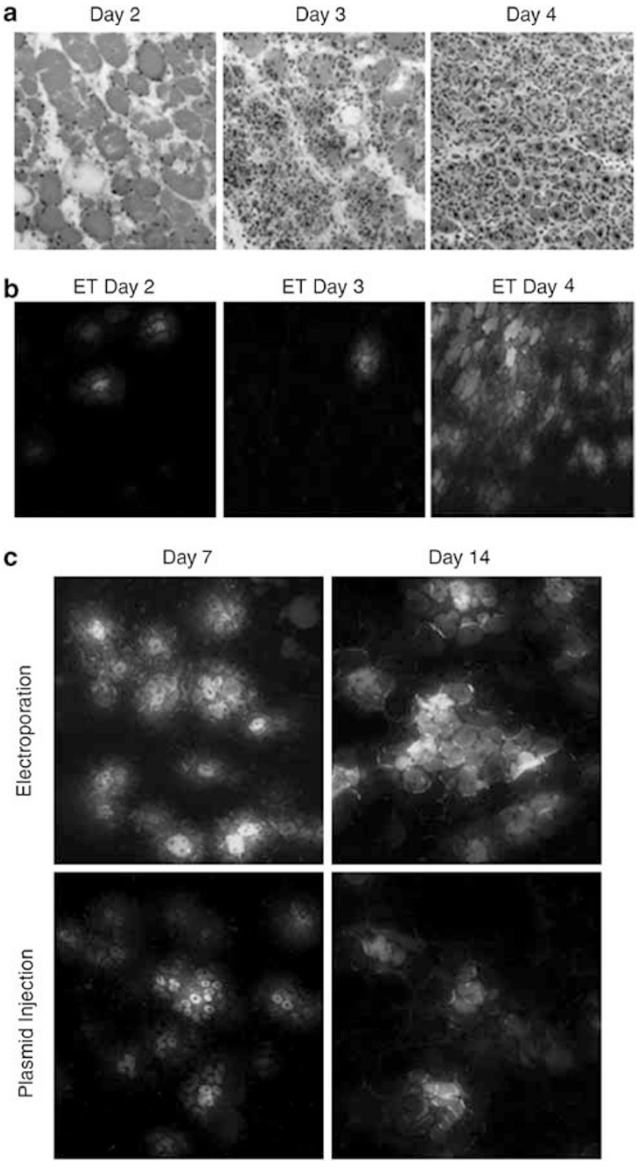

Figure 1 Representative photomicrographs of H&E-stained transverse TA muscle sections at 2, 3 or 4 days after myotoxic injury (**a**). Representative photomicrographs of EGFP in transverse sections of TA muscles 10 days after notexin-induced injury and i.m. injection of 80 μg of a plasmid encoding EGFP and electrotransfer (ET) at either 2, 3 or 4 days after injury (**b**). Representative photomicrographs of EGFP in transverse sections of TA muscles 7 or 14 days after notexin-induced injury and i.m. injection of 80 μg of a plasmid encoding EGFP alone or combined with ET, 4 days following the myotoxic injury (**c**).

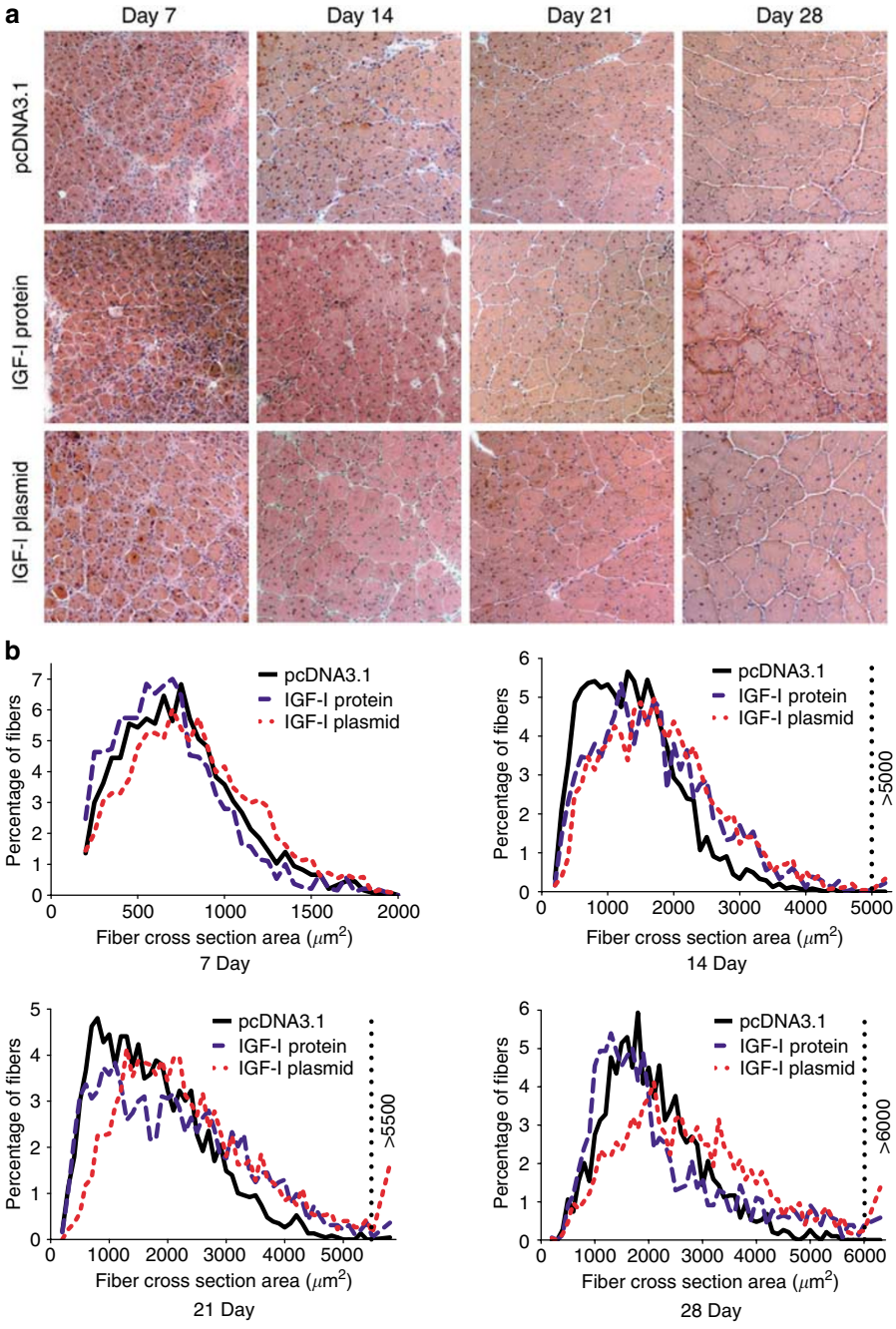

Figure 2 Representative photomicrographs of H&E-stained transverse TA muscle sections (**a**) and graphical representations (**b**) of myofiber CSA 7, 14, 21 or 28 days following notexin-induced injury. Four days after myotoxic injury, the TA muscle was injected with 80 μg of a plasmid encoding IGF-I followed by electrotransfer (IGF-I plasmid). Electrotransfer following injection of 80 μg of a non-coding plasmid was used for the control condition (pcDNA3.1). In addition, recombinant IGF-I protein was delivered systemically via mini-osmotic pump ($\sim$1.5 mg/kg/day), implanted 4 days after myotoxic injury.

$n = 2289$ fibers). Twenty-eight days post-injury, the median CSA of myofibers was significantly greater with electrotransfer of the plasmid encoding IGF-I (2444–2460 μm^2; $n = 1965$ fibers) compared to protein delivery (2042–2164 μm^2; $n = 1538$ fibers) or electrotransfer with pcDNA3.1 (2000–2083 μm^2; $n = 1866$ fibers).

ELISA, SDS-PAGE and Western blotting

Electrotransfer of a plasmid encoding IGF-I increased the concentration of IGF-I in the injured TA muscle and increased Akt phosphorylation (Figure 3). Conversely, systemic administration of IGF-I via mini-osmotic pump did not increase IGF-I levels in the injured muscle and did not activate IGF-I-mediated intracellular signals, such as Akt phosphorylation (Figure 3). We have demonstrated similar findings using a sensitive radioimmunoassay (RIA) for detection of IGF-I in diaphragm and other hindlimb muscles after the same dose of IGF-I delivery by mini-osmotic pump.[28,29]

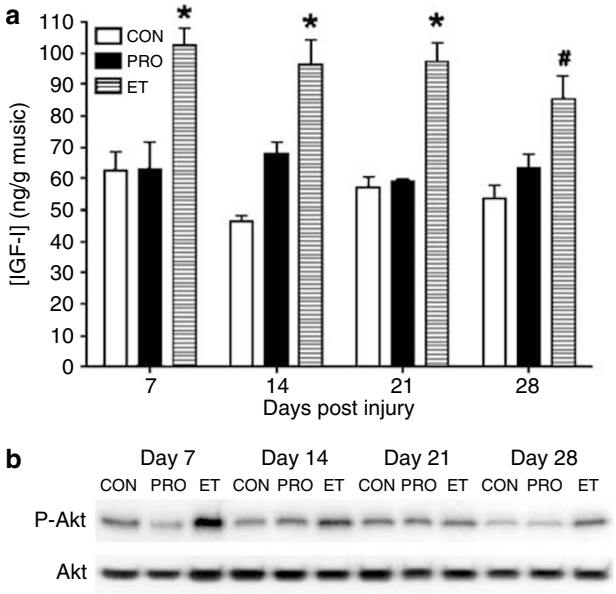

Figure 3 Concentration of IGF-I in TA muscles measured by ELISA (**a**). Representative Western blots of phospho-Akt(Ser 473) and Akt from TA muscles 7, 14, 21 or 28 days after notexin-induced injury (**b**). Four days after myotoxic injury, the TA was injected with 80 μg of a plasmid encoding IGF-I followed by electrotransfer (ET). ET following injection of 80 μg of pcDNA3.1 was used as the control condition (CON). Recombinant IGF-I protein was delivered systemically via mini-osmotic pump (~1.5 mg/kg/day), and implanted 4 days after myotoxic injury (PRO). *Significantly different from CON and PRO. #Significantly different from CON.

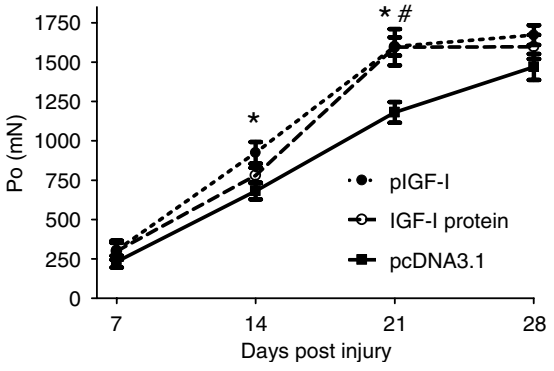

Figure 4 Mean data for tetanic force production of the TA muscle measured *in situ* at 7, 14, 21 or 28 days after notexin-induced injury. Four days after myotoxic injury, the TA was injected with 80 μg of a plasmid encoding IGF-I followed by electrotransfer (pIGF-I). Electrotransfer following injection of 80 μg of a non-coding plasmid was used for the control condition (pcDNA3.1). In addition, recombinant IGF-I protein was delivered systemically via mini-osmotic pump (~1.5 mg/kg/day), and implanted 4 days after myotoxic injury. *ET is significantly greater than CON. #PRO is significantly greater than CON.

Skeletal muscle contractile properties measured in situ

Maximum isometric tetanic force (P_o) was 36% greater 14 days post-injury following electrotransfer-assisted IGF-I gene transfer compared to controls ($P<0.05$; Figure 4). Conversely, systemic IGF-I protein administration had no effect on P_o compared to control at 14 days post-injury (Figure 4). At 21 days post-injury, tetanic force was 35% greater following both electrotransfer-assisted IGF-I gene transfer and IGF-I protein delivery compared to controls ($P<0.05$; Figure 4). Additional morphometric and contractile properties of TA muscles are reported in Table 1. At 14 days post-injury, electrotransfer-assisted IGF-I gene transfer increased twitch force (59%), maximal rate of twitch force development (54%) and specific force (29%) compared to controls ($P<0.05$), whereas IGF-I protein delivery had no effect on these parameters at this time point. At 21 days post-injury, electrotransfer-assisted IGF-I gene transfer and IGF-I protein delivery increased twitch force and the maximal rate of twitch force development compared to controls ($P<0.05$).

Table 1 Isometric contractile properties of mouse TA muscle measured *in situ* at various time points after notexin-induced injury following systemic administration of recombinant IGF-I protein, electrotransfer of plasmid DNA encoding for IGF-I or electrotransfer of a non-coding plasmid

Days post injury	*Muscle mass (mg)*	P_t *(mN)*	dP_{twitch}/dt *(mN/ms^{-1})*	sP_o *(kN/m^2)*
7				
pcDNA3.1	43±2	48±9	15±1	48±7
pIGF-I	46±3	78±15	16±1	63±11
IGF-I protein	42±3	75±18	16±1	64±9
14				
pcDNA3.1	47±3	205±23	35±3	117±8
pIGF-I	52±3	326±31[a,b]	54±5[a,b]	151±11[a]
IGF-I protein	50±2	225±18	39±3	128±7
21				
pcDNA3.1	57±3	338±30	55±4	176±16
pIGF-I	68±3	468±21[a]	71±4[a]	207±11
IGF-I protein	72±5[a]	458±32[a]	69±4[a]	195±5
28				
pcDNA3.1	61±3	407±30	67±5	235±21
pIGF-I	66±3	427±38	68±4	221±6
IGF-I protein	70±2	426±24	66±2	204±8

Abbreviations: dP_{twitch}/dt, maximal rate of force development during twitch response; IGF-I protein, systemic administration of recombinant IGF-I protein via mini-osmotic pump (1 mg/kg/day); P_t, maximal twitch force; sP_o, specific force (maximum tetanic force/muscle cross-sectional area); pcDNA3.1, electrotransfer of a non-coding plasmid; pIGF-I, electrotransfer of plasmid DNA encoding for IGF-I.
[a]Significantly different from pcDNA3.1.
[b]Significantly different from IGF-I protein; $n \geqslant 5$ mice in each group.

Discussion

Skeletal muscle has a remarkable regenerative capacity, but any impairment in the restoration of contractile properties after injury can lead to prolonged functional disability. Although it has been demonstrated that increased IGF-I levels improve skeletal muscle regeneration after various types of injuries, no study had compared the relative efficacy of various methods of IGF-I delivery to enhance functional recovery.[17,18,20–22] Our findings indicate that IGF-I hastens functional recovery after myotoxic injury, regardless of the route of administration, but that electrotransfer-assisted

plasmid-based gene transfer improved contractile function at an earlier time point after injury.

In a preliminary study, we established that 4 days after a notexin-induced injury was the earliest time at which we could transfect a large number of muscle fibers. Transfection efficiency was reduced greatly if plasmid injection and electrotransfer were performed 2 or 3 days after the notexin-induced injury. Complete degeneration of muscle fibers occurs 3 days after notexin injection. Therefore, the low transfection efficiency of gene transfer when attempted at or before 3 days could be due to breakdown of the transfected fibers, degradation of the plasmid and/or the relative difficulty of transfecting muscle precursor cells compared to myotubes or myofibers. Importantly, the earliest time at which we could get a large number of transfected muscle fibers was 4 days after notexin injection, which coincides with the initial emergence of immature (small) myotubes. This finding also supports the notion that myotubes are more easily transfected than muscle precursor cells.[30] In addition, the findings of the present study compared to those we have published previously confirm that the transfection efficiency of naked plasmid DNA injection without electrotransfer is higher in regenerating muscles than uninjured muscles, as noted by others.[23–27] We have also demonstrated that electrotransfer increases transfection efficiency in regenerating muscle, when performed at an appropriate time after injury.

Electrotransfer-assisted plasmid-based gene transfer increased maximum force production at 14 and 21 days post-injury, whereas systemic protein administration only increased maximum force at 21 days post-injury. IGF-I hastened the restoration of force-producing capacity and the rate of force development, effects that occurred earlier after plasmid-based gene transfer than with protein administration.

The IGF-I-induced changes in contractile properties were accompanied by morphological changes to the muscle fibers. IGF-I had no effect on myofiber CSA at 7 days post-injury, but at 14 and 21 days post-injury, plasmid-based gene transfer of IGF-I induced greater myofiber hypertrophy than systemic IGF-I protein administration. IGF-I plasmid-induced changes in myofiber CSA 28 days post-injury did not correlate with changes in contractile properties. This disparity between muscle function and morphological measurements (such as CSA or mass) during muscle regeneration has been observed before and may be attributed to the presence of developmental isoforms of contractile and regulatory proteins among other factors throughout the various stages of muscle regeneration.[31,32] It is possible that in response to sustained high levels of IGF-I other compensatory factors could be involved in the functional recovery in the latter stages of skeletal muscle regeneration.

Electrotransfer-assisted plasmid-based gene transfer increased IGF-I levels and activated downstream intracellular signals such as Akt phosphorylation, in the injured muscle. Interestingly, systemic administration of IGF-I via mini-osmotic pump did not activate IGF-I-mediated signalling cascades in the injured/regenerating muscle, but did increase tetanic force production compared to controls at 21 days post-injury, and so it did hasten functional recovery. We have demonstrated similar effects in previous studies, where recombinant IGF-I protein was delivered to mice at an identical dose via mini-osmotic pumps.[28,29] In these studies, systemic IGF-I protein administration induced morphological and functional changes, but IGF-I levels, as measured by RIA, were elevated in blood serum, but not in diaphragm or limb muscles.[28,29] Given its therapeutic potential to enhance restoration of muscle structure and function after damage, closer examination of the mechanisms of action of IGF-I at this dose and route of delivery is warranted. It should be noted that the hastened functional recovery after electrotransfer could be directly related to higher IGF-I levels in regenerating muscle.

Multiple transcripts of the IGF-I gene encode different isoforms, which arise from divergent promoter usage, splicing events and post-translational modifications.[33] Although various prepropeptides produce an identical mature IGF-I protein, these isoforms are postulated to have different effects on skeletal muscle. Specifically, divergence in the E-peptide region of IGF-I (which result in IGF-IEa or IGF-IEb isoforms) may dictate the potency of hypertrophic or proliferative responses.[34] Recently, it was demonstrated that viral-mediated overexpression of both IGF-IEa or IGF-IEb precursors induce skeletal muscle hypertrophy in young (growing) mice, whereas only IGF-IEa (and not IGF-IEb) induced hypertrophy in adult mice.[35] In the present study, we used IGF-IEa, as it is the most well-characterized isoform and has been consistently associated with skeletal muscle hypertrophy. It would be interesting to characterize the effects of IGF-IEb overexpression on the proliferation of myogenic cells in regenerating muscle. Although speculative, it is possible that the proliferative effects related to sustained expression of IGF-IEb may enhance early events in myogenesis, but inhibit later events (i.e. differentiation), thus interfering with functional restoration after injury.

In summary, we have demonstrated that IGF-I delivery hastens the functional recovery of skeletal muscle after myotoxic injury. Electrotransfer-assisted plasmid-based gene transfer of IGF-I was superior to systemic protein administration because it increased IGF-I levels in the injured muscle, activated IGF-I-mediated intracellular signals involved in hypertrophy (i.e. Akt phosphorylation) and induced a greater increase in myofiber CSA. Importantly, gene transfer of IGF-I hastened functional recovery earlier after injury (14 days), compared to protein administration (21 days). The results demonstrate that mode of delivery is an important variable when assessing the therapeutic potential of growth factors for promoting skeletal muscle regeneration.

Materials and methods

Experimental design

All procedures were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and conformed to the Guidelines for the Care and Use of Experimental Animals described by the National Health and Medical Research Council of Australia. Twelve- to fourteen-week-old male C57/BL10 mice were anesthetized with sodium pentobarbitone (Nembutal, Rhone Merieux, Pinkenba, Queensland, Australia, 60 mg/kg, intraperitoneal). The right hindlimb was shaved and a small portion of the TA muscle

surgically exposed and injected with 40 μl of notexin (Latoxan, Valence, France, 10 μg/ml in saline) using a 29-G fixed needle. After the intramuscular (i.m.) injection, the skin incision was closed with Michel clips (Aesculap, Tuttlingen, Germany).

Preliminary experiments were conducted to determine the earliest time at which the muscle could be adequately transfected after the myotoxic injury. Methodological details of plasmid injection and electrotransfer parameters have been described in detail previously.[23] A reporter plasmid encoding EGFP was injected and electrotransferred 2, 3 and 4 days after the notexin-induced injury. All of the TA muscles were harvested at 10 days post-injury. In order to determine whether electrotransfer was redundant in regenerating TA muscle, the plasmid encoding EGFP was injected alone or in combination with electrotransfer 4 days after the notexin-induced injury and the TA muscles were harvested 7 or 14 days post-injury.

Following the preliminary experiments, mice were randomly assigned to one of three experimental groups and for testing at either 7, 14, 21 or 28 days after notexin-induced injury to one TA muscle ($n \geqslant 5$ mice in each group). The injured TA muscles from the first experimental group were administered a single injection of plasmid DNA encoding IGF-I coupled with electrotransfer 4 days after notexin-induced injury. The other experimental group was administered recombinant IGF-I protein (IM050, GroPep, Adelaide, Australia, $\sim$1.5 mg/kg/day, subcutaneously) systemically via a mini-osmotic pump (model 1002; Alzet, Cupertino, CA, USA) implanted 4 days after notexin-induced injury, as described in detail previously.[28,29] At the completion of the treatment period, the osmotic pumps were aspirated to ensure that fouling had not occurred, and that the contents had been administered accordingly, as described previously.[28,29] The third group of mice served as controls, and received a single injection of non-coding plasmid DNA (pcDNA3.1) coupled with electrotransfer in the injured TA 4 days after notexin-induced damage.

Plasmid DNA preparation and muscle electrotransfer

A plasmid encoding EGFP (pEGFP-C1, Clontech, Palo Alto, CA, USA) was used during preliminary experiments to assess transfection efficiency. Subsequently, a mammalian expression vector-encoding rat IGF-IEa isoform was used (MGC 105288, American Type Culture Collection, Rockville, MD, USA). For all experiments, plasmid DNA was isolated by anion-exchange using Endotoxin free Giga kits from Qiagen (12391, Doncaster, Victoria, Australia) according to the manufacturer's instructions.

Four days after notexin-induced damage, mice were anesthetized (as described previously), the right TA muscle surgically exposed and injected with 30 μl of 0.5 U/μl hyaluronidase, and (2 h later) injected with plasmid DNA (40 μl, 2 μg/μl) using a 29-G fixed needle. Approximately 1 min after plasmid DNA injection, three 20 ms square wave pulses (75–100 V/cm) of 1 Hz frequency were generated using a Grass stimulator (Grass S88, Quincy, MA, USA) and delivered transcutaneously across the muscle using a pair of custom-built platinum plate electrodes attached to digital Vernier calipers. The polarity was then reversed and a further three pulses were delivered to the muscle.[23]

Skeletal muscle contractile properties measured in situ

The methods for *in situ* measurement of contractile properties of TA muscles from mice have been described in detail elsewhere.[23] Mice were anesthetized (as described earlier), the right hindlimb shaved and a single incision made to the anterior surface of the hindlimb to expose the TA muscle. The exposed tendon of the TA was cut several millimeters distal to the end of the muscle and tied securely to the lever arm of a dual-mode servomotor (Model 305B-LR, Aurora Scientific, Richmond Hill, Ontario, Canada). The foot of the mouse was immobilized by clamping it to the platform and a pin was passed behind the patellar tendon to immobilize the knee. The TA muscle was stimulated by supramaximal (10 V) 0.2-ms square wave pulses of 300 ms in duration and delivered via two wire electrodes adjacent to the femoral nerve. Optimum muscle length (L_o) was determined from maximum isometric twitch force (P_t) and maximum isometric tetanic force (P_o) was recorded from the plateau of a full frequency–force curve. Optimum fiber length (L_f) was determined by multiplying L_o by the TA L_f/L_o ratio of 0.6.[36]

Histology and immunofluorescence

Muscles were surgically excised, mounted in embedding medium, frozen in thawing isopentane and stored at -80°C. A portion of each frozen muscle sample was sectioned transversely (8 μm) through the mid-belly region on a cryostat microtome at -20°C. Muscle sections used for EGFP analysis were not fixed or mounted and were analyzed by fluorescence microscopy, as described previously.[23] Muscle sections were stained with hematoxylin and eosin (H&E) to determine general muscle architecture, and to determine the CSA of individual muscle fibers.[23] Median values for CSA were calculated from counting at least 200 individual muscle fibers per cross-section.

ELISA, SDS-PAGE and Western blotting

TA muscles were surgically excised and prepared according to methods described previously.[37] Total protein concentration of each sample was determined in triplicate by the method of Bradford. An equal amount of protein for each sample (50 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to a polyvinylidene difluoride membrane and blocked for 1 h in phosphate-buffered saline (PBS) containing 10% non-fat milk. Membranes were incubated overnight at 4°C with rabbit polyclonal antibodies specific for Phospho-Akt^{Ser473} or Akt (0.2 μg/ml; no. 9271 and no. 9272, Cell Signalling, Beverly, MA, USA), which were diluted in PBS plus 5% bovine serum albumin. Membranes were washed extensively and incubated with a horseradish peroxidase-conjugated anti-rabbit antibody for 1 h (AB324P, Chemicon, Temecula, CA, USA). Protein quantification was performed using an enhanced chemiluminescence immunodetection procedure as described previously.[38] IGF-I concentrations in TA muscle were measured using an enzyme-linked immunosorbent assay (ELISA). In a separate group of animals, TA muscles were surgically excised, rinsed with PBS to remove excess blood and homogenized in PBS, as described previously.[37] Samples were centrifuged at 5000 *g* and the

supernatant fractions were assayed for IGF-I by ELISA according the manufacturer's instructions (Quantikine IGF-I immunoassay, MG100, R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Individual variables were compared between groups using a one-way or two-way analysis of variance as appropriate. Bonferroni's *post hoc* multiple comparison procedure was used to detect differences between specific means. Significance was set at $P<0.05$. As muscle fiber CSA data were not normally distributed, 95% CIs of the median were used for assessing differences between groups. All other values are expressed as mean $\pm$ s.e., unless specified otherwise.

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